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CRE-Mediated transcriptional activation is involved in cAMP protection of T-cell receptor-induced apoptosis but not in cAMP potentiation of glucocorticoid-mediated programmed cell death

Lionel Müller Igaz [a], Damián Refojo [a,1], Mónica A. Costas [a,1], Florian Holsboer [b], Eduardo Arzt [a,1,*]

[a] *Laboratorio de Fisiología y Biología Molecular, Departamento de Biología, FCEN, Universidad de Buenos Aires, Ciudad Universitaria, Pabellon II, 1428 Buenos Aires, Argentina*

[b] *Max-Planck Institute of Psychiatry, Kraepelinstrasse 2–10, 80804 Munich, Germany*

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Abstract

Apoptosis of thymic cells induced by glucocorticoids (GC) and T-cell receptor (TCR) engagement are mutually antagonistic. We demonstrate that cAMP enhances GC and antagonizes TCR (anti-CD3) apoptosis on the same cell (DO-11.10 and 2B4.11 T-cell hybridomas). We analyzed the activity of several transcription factors in this cAMP dual, stimulus-dependent, regulatory action. Anti-CD3 increases kB-activity which is inhibited by CPTcAMP or dexamethasone (DEX), supporting the proapoptotic role of NFkB on TCR-induced apoptosis. Anti-CD3 not only increases kB- but diminishes GC response element (GRE)-activity induced by DEX, suggesting that TCR-mediated blockade of GC-induced apoptosis involves not only the proposed antiapoptotic action of NF-kB on GC, but also the inhibition of GRE-regulated proapoptotic genes. To test the involvement of CRE-driven transcription in the cAMP dual apoptotic regulation, cells were transfected with a CRE decoy DNA oligomer. Blockade of CRE transactivation with decoy targeting of CRE completely blocked the protection of TCR-induced apoptosis by cAMP, while it did not modify the enhancement by cAMP on GC-induced apoptosis. We show that CRE-binding factors have a definite role in T-cell apoptosis: they are involved in cAMP protection of TCR- but not in cAMP potentiation of GC-induced apoptosis.

Keywords: Glucocorticoids; Cyclic AMP; T cell receptor; cAMP responsive element binding protein; Apoptosis

1. Introduction

Apoptosis in T lymphocytes occurs in a variety of processes, e.g in deletion of autoreactive T cells during thymic maturation [1,2], in cells attacked by cytotoxic T lymphocytes [3], in activation-induced cell death and when using antibodies specific for the T-cell receptor (TCR) antigen complex [4]. A well studied model of apoptosis is the glucocorticoid (GC)-induced programmed cell death in immature thymocytes [5–7], which has also been shown to occur in vivo in rats [7] and chickens [8].

Apoptosis plays a role in the deletion of peripheral T cells and selection process of thymocytes during their development in the thymus, and can be induced

* Corresponding author. Fax: +54-11-4576-3321.
E-mail address: earzt@bg.fcen.uba.ar (E. Arzt).

[1] Members of the Argentine National Research Council (CONICET).

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either by cellular activation or by exposure to GC [9,10]. The default pathway for thymocytes bearing TCRs with subthreshold avidity for self antigens is death (death by neglect), which is mostly induced by GC. Both the activation and GC-mediated apoptosis seem to be responsible for thymocyte selection. Based on T-cell hybridoma models it has been proposed that the mutual antagonism among activation-induced and steroid-mediated pathways play a critical role in the selection process [5,8–11]. In the periphery, TCR signaling initiates the cellular immune response activating T cells. In turn, TCR stimulation of already activated mature T cells can undergo apoptosis. This process is denominated activation-induced cell death. This is also inhibited by GC, indicating that GC also mediate survival or apoptosis in mature T cells [12]. Understanding the molecular basis for T-cell apoptosis has been greatly aided by the development of cell culture systems, such as T-cell hybridomas, amenable to genetic manipulations.

The involvement of cAMP in programmed cell death has been studied in different systems, and is far from clear in T cells yet. Some controversial results have been reported. It induces apoptosis in a rat myeloid leukemic cell line [13]. In mouse fetal thymus organ cultures the cAMP signaling pathway blocks differentiation of thymocytes at an early maturation stage [14]. In primary cultures of mice thymocytes cAMP was also shown to potentiate GC-induced apoptosis [15]. In B10 T-cell hybridomas, cAMP-elevating agents have been shown to prevent TCR-induced apoptosis with minimal inhibition of interleukin-2 production [16] and antagonism of Fas-dependent activation [17]. cAMP analogs induced DNA fragmentation in 9C12.7 but not in other T-cell hybridomas and did not influence GC-induced apoptosis in all these cell lines [18].

cAMP transcriptional activity is essentially mediated by CREB (cAMP-responsive element binding protein), a 43 kDa basic/leucine zipper transcription factor that binds to the octanucleotide CRE element (TGANNTCA) both as homodimer and as heterodimer in conjunction with other members of the CREB/ATF superfamily of transcription factors such as cAMP-responsive element modulator (CREM) [19]. The mammalian NF-kB transcription factors, which include p50/p105, p52/p100, RelA (p65), c-Rel and RelB, play important roles in the regulation of immune responses, cellular proliferation and cell death (reviewed in [20]). Transcriptional activation or repression of NF-kB target genes requires the binding of NF-kB dimers to kB DNA-binding sites. In most cells, the transcriptional activity of NF-kB proteins is controlled at the posttranslational level by association with members of a family of inhibitory proteins (IkBs). The glucocorticoid receptor (GR) acts on target cells primarily through transcriptional activity through binding of the GR to the DNA glucocorticoid responding elements (GRE) [21]. The cross-talk between transcription factors modulates the responsiveness of cells to different stimuli. Cross-talk of GR with AP-1 and NF-kB has been shown to be the molecular substrate of GC inhibition of cytokine expression and action [22–26]. A complex cAMP-dependent GR regulation, involving changes in receptor transcript level, ligand binding and phosphorylation status has been described [27–29]. For these transcription factors, e.g., CREB, NF-kB and GR, both pro- and antiapoptotic actions have been described in different cell types [11,30–33].

The putative integrative regulation of cAMP-mediated pathways in the GC/TCR-induced apoptosis has not yet been studied. In the present report we address this question as well as the involvement of the main transcription factors implicated in these pathways making use of the T-cell hybridoma models utilized to define the mutual antagonism among activation-induced and steroid-mediated pathways.

2. Materials and methods

2.1. Materials

Unless stated, reagents used in the present work were obtained from Sigma Chemical Co. (St. Louis, MO).

2.2. Cell culture and stimulation

Cell culture procedures have been previously described [26,34]. DO-11.10 mouse T-cell hybridoma (generously provided by Dr. Makoto Iwata, Mitsubishi Kasei Institute of Life Sciences, Tokyo, Japan) was grown in Dulbecco's modified Eagle's medium

(DMEM) (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated FCS (Gibco, Paisley, UK), penicillin (100 units/ml), streptomycin (100 μg/ml), 3 mM L-glutamine, 50 μM β-mercaptoethanol and 100 mM non-essential amino acids (Gibco, Paisley, UK). 2B4.11 mouse T-cell hybridoma (generously provided by Dr. Jonathan Ashwell, NIH, USA) was grown in RPMI 1640 (Gibco Laboratories) supplemented with 10% heat-inactivated FCS, 4 mM L-glutamine, 50 μM β-mercaptoethanol, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. The same medium as for cell maintenance was used for cell growth and plating before the experiments took place.

DO-11.10 and 2B4.11 cells (10^5 cells/ml) were activated with plate-bound 145.2C11 anti-CD3 antibodies (Pharmingen, USA), coated overnight at 4°C in Tris–HCl 40 mM buffer (pH 9.5). The following substances were also used: dexamethasone (DEX), a synthetic GC; CPTcAMP, dibutyryl-cAMP and 8-Br-cAMP, cell-permeable analogs of cAMP; forskolin, an adenylyl cyclase activator and isoproterenol, a β-adrenergic agonist (Research Biochemicals International, Natick, MA). After treatments, cells were harvested and analyzed to assess cell death (acridine orange staining, FACS and DNA fragmentation), and luciferase (LUC) activity. In some experiments, transfection of reporter plasmids or dsDNA oligonucleotides was performed prior to treatment.

2.3. Acridine orange/ethidium bromide staining

Mortality was evaluated adding fluorescent dyes that bind to DNA and subsequently examining the cells under an ultraviolet light microscope (Leica, Germany). At different times post-treatment (as indicated), 100 μl of cell suspension and 100 μl of a 1:1 PBS solution of acridine orange 1 mg/l and ethidium bromide 20 mg/l were mixed and immediately observed under ultraviolet light. Ethidium bromide only enters into non-viable/death cells and stains chromatin a dark orange color. Acridine orange penetrates in viable cells and turns green when it intercalates with DNA. At least 200 cells per condition were counted.

2.4. Flow cytometry

Apoptosis was also evaluated by double staining with propidium iodide and annexin V-FITC (Bender MedSystems, Vienna, Austria). After the different treatments, 5×10^5 cells were washed once with PBS, resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) and incubated on ice in darkness for 30 min with 2 μl of annexin V-FITC 20 mg/ml and 2 μl of propidium iodide (1 mg/ml stock). Samples were washed three times with 2 ml of binding buffer and then analyzed by flow cytometry in a Cytoron Absolute (Ortho Diagnostic Systems, New Brunswick, NJ, USA) cytometer equipped with a 488 nm argon laser. Data were obtained with ImmunoCount (Ortho Diagnostic Systems) software.

2.5. DNA laddering apoptosis assay

Each cell suspension (5×10^6 cells) was washed twice with PBS and the cell pellet was resuspended in 800 μl of lysis buffer (0.2% Triton X-100; 10 mM Tris–HCl, pH 7.5; 10 mM EDTA) [26]. After 10 min on ice, the lysate was centrifuged (14 000 rpm) for 10 min at 4°C and a phenol-chloroform/isoamyl alcohol (24:1) extraction of the supernatant was performed. The aqueous phase was precipitated with sodium acetate 30 mM and 0.7 volumes of isopropanol. The pellet was washed with 80% ethanol, dried and resuspended in 100 μl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8). Samples were treated with RNAse A (0.5 mg/ml) for 1 h. at 37°C, then a phenol-chloroform/isoamyl alcohol extraction was performed and the aqueous phase was precipitated with 2 volumes of ethanol. The nucleic acid pellet was resuspended in 40 μl of buffer TE, and electrophoresis was carried out in a 2% agarose gel in TBE buffer; DNA was visualized by ethidium bromide staining. A 123 bp marker (Gibco BRL, Grand Island, NY, USA) was used in parallel as an indicator of molecular mass.

2.6. Plasmids, transfections, and assay of luciferase activity

Reporter plasmids for GRE (MTV-LUC provided by Dr. R. Evans, Howard Hughes Medical Institute, La Jolla, CA, USA), CRE (ΔMTV-5CRE-LUC pro-

vided by Dr. Dietmar Spengler, Max Planck Institute of Psychiatry, Munich, Germany) and kB sites (kB-LUC provided by Dr. M. Bell, Mayo Clinic, Rochester, MN, USA) have been described elsewhere [35–38].

DO.11-10 and 2B4.11 cells were transfected by electroporation, according to a described method [26,39], with small modifications. Briefly, cells cultured in maintenance medium were washed with PBS and the cellular pellet was resuspended in electroporation medium (DMEM with 3 mM L-glutamine and 50 μM β-mercaptoethanol). Cells (10^7 in 0.2 ml) were transferred to a 4-mm-gap electroporation cuvette (Bio-Rad Laboratories, Richmond, CA, USA) and 20 μg of the indicated reporter plasmid was added, followed by a 10-min incubation at room temperature. Transfection was carried out using an electroporation system Gene Pulser II (Bio-Rad Laboratories) at 250 V, 975 mF. Electroporated cells were replated in maintenance medium and incubated immediately with the different agents. After 6 or 24 h, cells were harvested and extracts were assayed for LUC activity [26] using a Promega (Madison, WI, USA) kit. Briefly, 20 μl of the extract were incubated with 100 μl of the Luciferase Assay Buffer in partial darkness and immediately assessed for luciferase activity in a Junior luminometer (Berthold, Germany). In order to correct for variations in the total number of harvested cells during extract preparation, relative luminescence units (RLU) were normalized with respect to the total protein content of each extract, according to the Bradford method [40].

2.7. Decoy targeting

DO.11-10 cells were transfected with dsDNA oligonucleotides using the same protocol as for plasmid transfection, with minor modifications. These oligonucleotides are specifically designed to bind to particular transcription factors and are used to dampen the content of these factors within the cell [41]. Due to the transfection procedure, which involves different cell batches for each DNA transfected and increased basal mortality levels, some differences in basal cell death number are observed in decoy targeting experiments. The indicated amount of oligonucleotides were transfected and cells were incubated for 1 h at 37°C, 5% CO_2 before exposing them to the different treatments. An oligomer with the CRE site (sequence: 5′-AGCTTAGGGCTCGT**TGA**CG**TCA**CCAAG-3′) or a control oligonucleotide (5′-AGCTTAGGGCTCGT**TGA**CG**TC<u>T</u>**CCAAG-3′) were used. This latter has the same base composition but an inverted base pair, which renders it unable to bind CREB proteins [38]. In preliminary experiments we proved the ability of the CRE oligonucleotide to block CRE transcriptional activity, in contrast to the control oligonucleotide. For convenience we will use the term CREB to refer to the CREB superfamily of transcription factors (which bind to CRE).

2.8. Statistics

Statistics were performed using one-way analysis of variance (ANOVA) in combination with Scheffé's test. Results are expressed as mean ± S.E.M.

3. Results

3.1. cAMP has an opposite action on GC and TCR-induced apoptosis in the same cell

In order to assess the role of cAMP as a modulator of GC and TCR-mediated programmed cell death, two types of mouse T-cell hybridomas were used: DO-11.10 and 2B4.11. Unless specified, the results were identical in these two cell lines. As shown for DO-11.10 cells, anti-CD3 (which mimics TCR engagement) or DEX treatment induced apoptosis, as evaluated by acridine orange staining after 16 h (Fig. 1A). The cAMP analog CPTcAMP potentiated the effect of DEX, but significantly decreased anti-CD3 triggered apoptosis (Fig. 1A); similar results were obtained with forskolin (an adenylyl cyclase activator) and isoproterenol (a β-adrenergic agonist) (not shown). CPTcAMP had no effect by itself on apoptosis, and the mutual inhibition of TCR- and GC-mediated apoptosis was also evident (Fig. 1A). At these doses, DEX and CPTcAMP elicited a quantitatively similar protection. Both regulatory effects of cAMP were also confirmed by flow cytometry with annexin V-FITC staining (an early marker of apoptosis) (Fig. 1B) and by agarose gel electrophoresis (to determine the DNA laddering pattern observed after internucleosomal cleavage) (not shown).

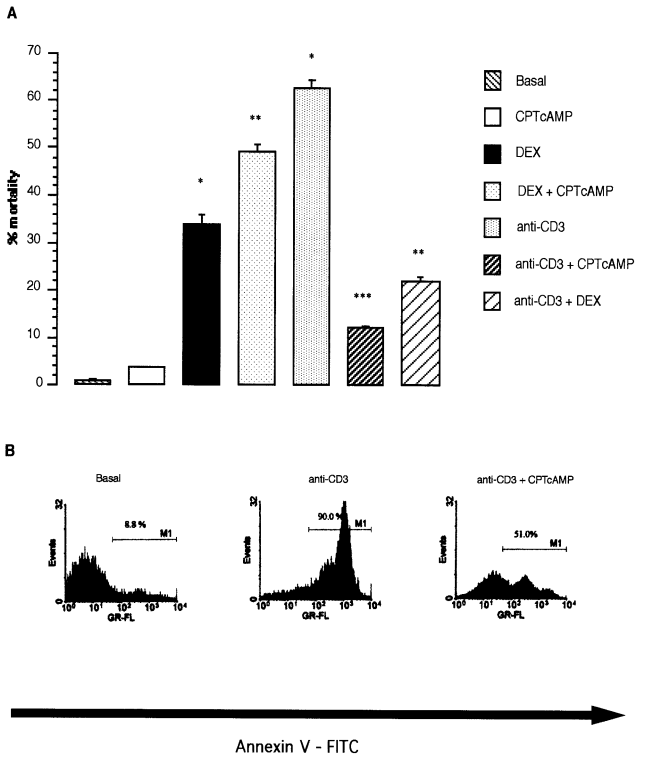

Fig. 1. cAMP differentially modulates GC- and TCR-induced apoptosis on the same cell. (A) DO.11-10 cells were incubated with optimal doses of DEX (1 μM), CPTcAMP (500 μM) and anti-CD3 (500 ng/ml), as indicated. Cell mortality percentage was assessed 16 h after treatment with acridine orange plus ethidium bromide staining and fluorescence microscopy, as described in Section 2. The error bars correspond to the mean of quadruplicate samples ± S.E.M. Similar results were obtained in three independent experiments. $*P < 0.001$ with respect to basal condition; $**P < 0.001$ with respect to DEX on its own; $***P < 0.001$ with respect to anti-CD3 on its own (ANOVA with Scheffé's test). (B) DO.11-10 T-cell hybridoma cells were stimulated for 16 h as in A. Subsequently, cells were analyzed by flow cytometry to assess annexin V-FITC binding. Simultaneously, cells were incubated with propidium iodide to evaluate cell viability (not shown). Representative fluorescence histograms from one of two separate experiments with triplicates are shown.

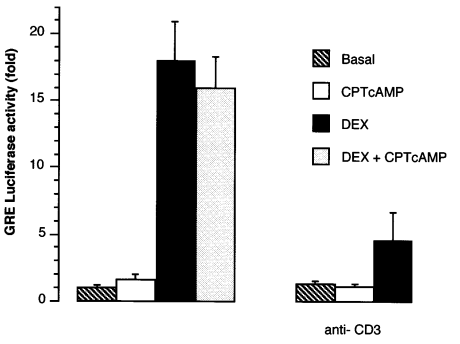

Fig. 2. Anti-CD3 treatment inhibits DEX-mediated increase of GRE-mediated transcription. 10^7 DO-11.10 cells were transfected with 20 μg of GRE-LUC and stimulated with 100 nM DEX, 500 μM CPTcAMP and 500 ng/ml anti-CD3. After 24 h incubation, cells were harvested for extract preparation and LUC activity determination; fold activity with respect to basal levels is shown. LUC activity is the mean of three normalized measurements ± S.E.M. Similar results were obtained in four independent experiments.

In these experiments we demonstrate, for the first time, that cAMP exerts a dual regulatory action on the same cell, depending on which is the stimulatory pathway for apoptosis.

3.2. GRE and kB activity during T-cell hybridoma apoptosis triggered by GC, TCR and cAMP

Since cAMP-modulated factors could be playing a role in a cross-talk with signals that trigger other important transcriptional regulators (such as activated GR and NF-kB), DO-11.10 cells were transfected with reporter plasmids to assess transcriptional activity levels of factors that bind to GRE and kB-sites. MTV-LUC (a LUC construct containing four tandem repeats of GRE) [35] was used to evaluate modulation of GR transcriptional activity. DEX treatment of T-cell hybridoma strongly increased LUC expression mediated by GREs, while anti-CD3 stimulation inhibited DEX-mediated GRE-transactivation. CPTcAMP did not modify GRE-driven activity (Fig. 2). To study NF-kB transcriptional activity, we used a kB-LUC plasmid containing 3 kB sites from the HIV enhancer coupled to luciferase [36]. kB activity levels were induced by anti-CD3 stimulation and inhibited by treatment with either DEX or CPTcAMP (Fig. 3), while both together had not further additive effects (not shown). Similar effects were observed when cells were stimulated only with DEX, CPTcAMP or a combination of the two.

3.3. CRE-driven transcription exerts an anti-apoptotic regulation. Role of CRE-binding factors as inhibitors of TCR-induced apoptosis

To further analyze the dual effect of cAMP signal transduction pathways on GC- and TCR-induced apoptosis, we transfected DO-11.10 cells with a

CRE-LUC reporter plasmid (ΔMTV-5CRE-LUC), which has deleted the GRE-containing sequences but possesses five CRE tandem repeats [37,38] (Fig. 4). As expected, all treatments with CPTcAMP enhanced CRE-driven transcription, either with or without DEX or anti-CD3 stimulation.

Anti-CD3 stimulation decreased basal levels of CRE activity by 70%. Both co-treatment with DEX or the cAMP analog blocked this inhibition, up to basal (in the case of DEX) or even higher levels (Fig. 4). CPTcAMP induction of CRE activity was weaker when presented in combination with anti-CD3. DEX partially inhibited the effect of the cAMP analog when presented without anti-CD3 stimulation. These results show that anti-CD3 treatment decreases basal levels of CRE-driven activity, while inhibitors of TCR-mediated cell death reverse these effects.

These results suggested a putative protective effect for CRE-binding factors in TCR-induced apoptosis. To test this hypothesis, we transfected DO-11.10 cells with dsDNA oligonucleotides carrying the CRE consensus sequence (an approach termed 'decoy targeting'), and evaluated cell death after the different treatments. Blockade of CRE-driven transcriptional activity completely blocked cAMP protection of TCR-induced apoptosis, while an identically composed scrambled control oligonucleotide had no effect (Fig. 5A). We also analyzed if CRE-binding factors participated in CPTcAMP potentiation of GC-induced cell death. As shown in Fig. 5B, the same CRE oligonucleotide did not change the enhancement exerted by the cAMP analog on DEX-mediated apoptosis; the fold increase of mortality was identical in control and CRE oligonucleotides (comparing DEX versus DEX+CPTcAMP treatments). Other experiments with different doses of oligonucleotides had a similar, dose-dependent result (not shown).

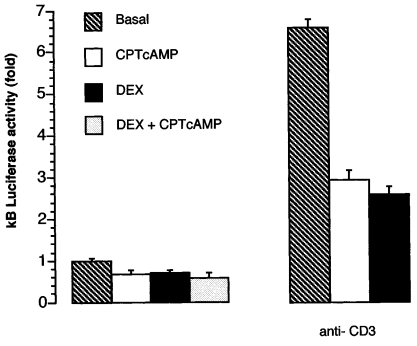

Fig. 3. Enhancement of kB-driven transcriptional activity by anti-CD3 treatment and inhibition by co-stimulation with DEX or CPTcAMP. 10^7 DO-11.10 cells were transfected with 20 μg of kB-LUC and stimulated with 100 nM DEX, 500 μM CPTcAMP and 500 ng/ml anti-CD3. After 6 h incubation, cells were harvested for extract preparation and LUC activity determination; fold activity with respect to basal levels is shown. LUC activity is the mean of three normalized measurements ± S.E.M. Similar results were obtained in three independent experiments.

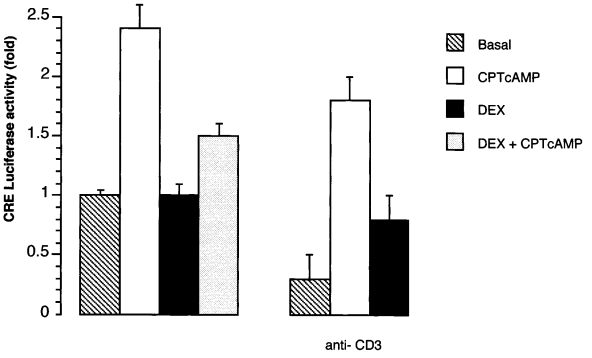

Fig. 4. CRE transcriptional activity is inhibited by anti-CD3 treatment. 10^7 DO-11.10 cells were transfected with 20 μg of ΔMTV-5CRE-LUC plasmid and stimulated with 100 nM DEX, 500 μM CPTcAMP and 500 ng/ml anti-CD3. After 6 h incubation, cells were harvested for extract preparation and LUC activity determination; fold activity with respect to basal levels is shown. LUC activity is the mean of three normalized measurements ± S.E.M. Similar results were obtained in four independent experiments.

4. Discussion

In the last 10 years, a significant amount of work has been done to understand the regulatory mechanisms underlying thymic and T-cell apoptosis, mainly using T-cell hybridomas as cellular models [10,11, 39,42,43]. In this work we report for the first time the dual effect that cAMP exerts on apoptosis in the same cell, using two models, DO.11-10 and 2B4.11 T-cell hybridomas, and we show that CRE-binding factors have a definite role in T-cell apoptosis: they are involved in cAMP protection of TCR- but not in cAMP potentiation of GC-induced apoptosis.

We also analyzed the involvement of other transcription factors, performing functional reporter assays which give a clear insight on the transcription status of the factors. The role of NF-kB in the reg-

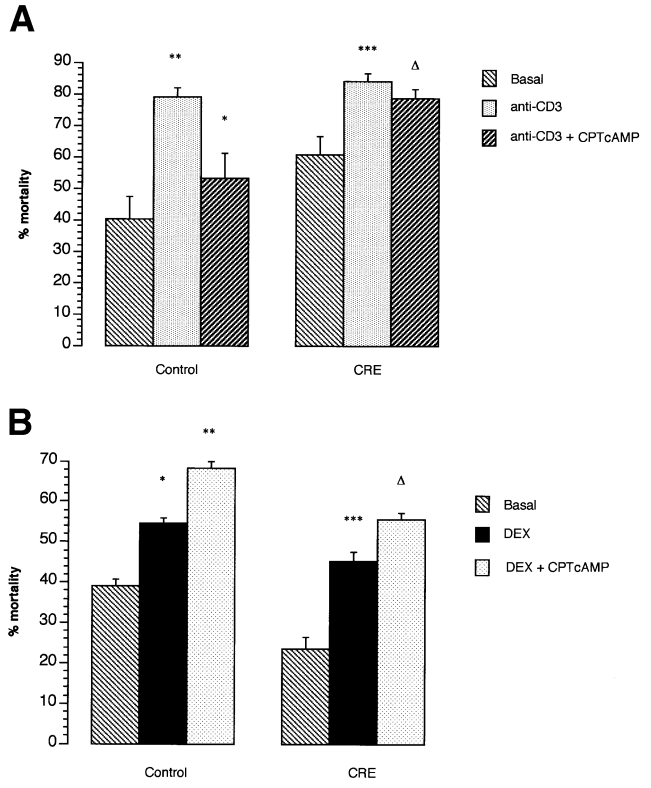

Fig. 5. Decoy targeting of CRE-binding factors with CRE oligonucleotide show CREB involvement in cAMP protection of TCR-induced apoptosis but not in cAMP potentiation of GC-mediated programmed cell death. DO.11-10 cells were transfected with 1.5 μg of dsDNA oligonucleotides containing CRE or control sequences (see Section 2). After 1 h incubation at 37°C, cells were stimulated with plate-bound anti-CD3 500 ng/ml, CPTcAMP 500 μM or DEX 100 nM, as indicated. Sixteen hours later cell mortality percentage was assessed with acridine orange plus ethidium bromide staining. The error bars correspond to the mean of triplicate samples ± S.E.M. Similar results were obtained in three independent experiments. Differences in basal levels are due to the use of different cell batches for each transfected DNA. Higher basal mortality, compared to Fig. 1, is due to the transfection procedure. (A) $^{*}P<0.05$ with respect to control oligo/anti-CD3; $^{**}P<0.01$ with respect to control oligo/basal condition; $^{***}P<0.01$ with respect to CRE oligo/basal condition; $^{\Delta}P<0.05$ with respect to CRE oligo/basal condition and similar to CRE oligo/anti-CD3 (ANOVA with Scheffé's test). (B) $^{*}P<0.01$ with respect to control oligo/basal condition; $^{**}P<0.01$ with respect to control oligo/DEX condition; $^{***}P<0.001$ with respect to CRE oligo/basal condition; $^{\Delta}P<0.05$ with respect to control oligo/DEX condition (ANOVA with Scheffé's test).

ulation of apoptosis is still under evaluation. In some models has been shown to be proapoptotic, whereas in others an antiapoptotic action has been demonstrated [32,39,44,45]. For example, downregulation of kB activity in T A1.1 hybridoma using an inhibitory peptide results in a significant decrease in Fas ligand expression and apoptosis in response to TCR ligation [46]. A recent report using T-cell hybridoma 2B4.11 as a model shows that this nuclear factor can perform both functions in the same cell, depending on the stimuli. In that study, blocking of NF-kB activity was shown to inhibit TCR-mediated apoptosis, while potentiating GC-induced apoptosis [39]. kB transcriptional activity has not been previously evaluated in these cells. Our results, which show an increase in kB activity by anti-CD3, are in line with this proapoptotic role of NF-kB by TCR-engagement. This recent study performed by Scheinman's group lead them to propose that NF-kB protection of GC-mediated apoptosis would represent a mechanism by which thymocytes survive during positive selection [39]. In order to induce apoptosis, GC should not only induce apoptosis-promoting genes but also induce genes (such as IkB) that block apoptosis inhibitors. The results of our transfection experiments with GRE- and kB-reporter plasmids (Figs. 2 and 3), showing that TCR engagement increases kB- and diminishes GRE-driven activity induced by DEX, suggest that TCR-mediated blockade of GC-induced apoptosis probably involves not only the previously demonstrated antiapoptotic action of NF-kB on GC and its proapoptotic activity on TCR ligation without GC, but also the inhibition of GRE-regulated proapoptotic genes. In fact in knock-in mice for the GR, it has been shown that GR transactivation is essential for the proapoptotic action of GC on thymocytes [47]. In addition, we observed that cAMP inhibited both basal and TCR-induced kB transactivation. These results are in agreement with previous publications. In human endothelial cells it has been shown that cAMP inhibits NF-kB-mediated transcription [48,49], while in T cells it has been shown that cAMP antagonizes Fas dependent activation induced cell death accompanied by a downregulation of NF-kB levels shown by gel shift [17] and that TCR-coupled Fas ligand expression and apoptosis was inhibited by cAMP [50]. In that paper a series of experiments in other cell lines suggested that the inhibition of Fas ligand expression by cAMP in T-cell hybridomas may be correlated with a specific inhibition of NF-kB [50]. The concomitant inhibition by cAMP of TCR-induced cell death and

kB transcriptional activity that we describe for the first time in a T-cell model, further supports the proapoptotic role that NF-kB may exert on TCR-induced apoptosis.

Studies performed during the 1970s presented the first evidence that cAMP promotes apoptosis. cAMP pharmacological agonists are also cytotoxic in lymphoid cell lines, and stimulate DNA fragmentation in thymocytes [16]. More recent work has extended these observations to other cellular models, including immortalized primary granulosa cells, human mammary carcinoma cells, and a variety of normal and transformed T and B cells [51,52]. According to our studies, the cAMP signaling pathway has no effect per se in T-cell hybridoma apoptosis; moreover, cAMP effects are most likely context-dependent. This is further indicated by evidence showing that in other models cAMP can block apoptosis. The best known example is probably the effect of cAMP on neurons, where it inhibits apoptosis triggered by ex vivo culture, nerve growth factor depletion or extracellular potassium [53]. It also prevents spontaneous apoptosis of aged neutrophils, ovary follicles [54] and, as shown in the present report and others, TCR-activated T cells [16,18,55]. To this day, it is not known if the same molecular targets are responsible for the apoptosis suppressing and promoting roles of cAMP.

CRE-binding factors are of major significance in current molecular physiology, in particular the CREB transcription factor. There is evidence that within the nervous system CREB plays a key role in the regulation of apoptosis. In addition, studies using CREB null mice or transgenic mice expressing a dominant negative of CREB suggest that it is necessary in thymus for the proper maturation of T lymphocytes [56,57]. In this work we demonstrate that CREB proteins are key mediators in the protection exerted by cAMP signaling on TCR-induced apoptosis (Fig. 5A). The molecular targets of CREB transcriptional regulation remain unknown; they may include several antiapoptotic genes which are reportedly up-regulated by cAMP through CREs in their promoters (such as bcl-2 family members mcl-1, bcl-xL and bcl-2 [41,58,59]) or FasL, which is also regulated by cAMP and is critical for T-cell apoptosis [17,50].

The experiments shown in Fig. 5 also establish that CRE-driven transcription is essential for cAMP action on TCR-mediated apoptosis, but has no apparent effect on the potentiation by cAMP of GC-dependent cell death. Interestingly, while CPTcAMP synergizes with DEX in the induction of apoptosis, DEX blocks CPTcAMP induction of CRE activity. A hypothetical explanation would be that GR interacts with a repressive CRE binding protein to promote apoptosis. Although in this study we focus on the cross-talk at the transcriptional level, GR and cAMP pathways may regulate each other upstream in the signal cascade, as for example recently described for the MAPK pathway in the GR/TCR antagonism [43]. The variations in mortality basal levels after CRE transfection may be due to the fact that different batches of cells were used in each electroporation (as stated in Section 2), and/or that competition for common coactivators (e.g., CREB binding protein, CBP) is taking place (Fig. 5). In addition, CREB or CBP very likely may be involved in the cAMP inhibition of NF-kB transactivation following TCR stimulation discussed above.

During stress, a number of hormones and neurotransmitters are released, in order to modify the system equilibrium toward a new condition where it can deal with the novel situation. Both systemic adrenaline and locally released noradrenaline participate in this mechanism, acting through adrenergic receptors. These act mainly by modulating the levels of cAMP, hence emphasizing a putative modulatory role for substances that transduce signals through this second messenger. Lymphoid tissues such as spleen and thymus, in particular, are abundantly innervated by adrenergic fibers. This is especially true during development, when it is believed that they may play a role in the differentiation and survival of developing T cells [60,61]. Furthermore, the potentiation of GC-mediated apoptosis with cAMP observed in this study and others [15] suggests a possible role of cAMP-elevating agents in thymic involution observed during stress.

Based on these results, further investigation will be required to determine the targets of GC, cAMP and TCR ligation in the development of thymocytes and T cells in primary cultures. Also, whether this dual modulatory stimulus-dependent role of cAMP/CRE in cell survival takes place in other cell types requires further study.

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